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Structure-function Relationships in the Evolution of Elastin

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The evolution of the structure of the rubber-like protein elastin, found in connective tissues which are subjected to periodic physiological stress, was studied with respect to its phylogenetic distribution, fiber morphology and arrangement, response to deformation, and amino acid composition. Aortae and other tissues from several vertebrates and invertebrates were examined for the presence of elastin, which was defined on the basis of a characteristic amino acid composition, the presence of the unique crosslinks desmosine and isodesmosine, and by histologic criteria. The protein was present in all vertebrates except the primitive jawless fishes and was absent from all invertebrates which were examined. In addition, the morphology of aortic elastin fibers differed markedly among the vertebrate families.

Biochemical analysis revealed increases in both the degree of crosslinking and hydrophobicity in elastins from higher vertebrates (mammals, birds) as compared to those from bony fish. Mammalian elastin displayed an increased tendency toward coacervation (polymerization into aggregated structures) at 37°C and behaved differently from a conventional elastomer when stretched in a microcalorimeter. Selection for an increasingly hydrophobic elastin appears to have paralleled the development of a highly-pressurized, closed circulatory system in homeothermic animals.

The data do not support a common genetic origin for elastin and other connective tissue proteins. Significant variations in amino acid composition among aortic elastins from different species, however, indicate that genetically distinct elastin types could have arisen by divergence from a common ancestral gene.

In previous studies we have discussed several aspects of the evolution of the rubbery connective tissue protein elastin [1–4]. The occurrence of this protein was coincident with the appearance of the efficient, highly pressurized circulatory systems common to all vertebrates except cyclostomes. The morphology and disposition of elastic fibers within aortae revealed in some cases apparently adaptive variations that were very striking. Reconstruction of the amino acid composition of an hypothetical, ancestral elastin has shown that this protein did not differ markedly in composition from that of present-day mammals. However, there has been a positive selection toward increasing hydrophobicity, as reflected by a greater proportion of hydrophobic to polar residues, in the higher vertebrates.

The participation of hydrophobic interactions has been demonstrated for 2 physical phenomena which may be unique to elastin: elasticity and coacervation/fibril formation [5–7]. Elastins which displayed variations in the ratio of apolar to polar amino acids and in the degree of crosslinking provided an opportunity for studying their relative behavior when subjected to conditions favoring coacervation and when stretched in a microcalorimeter. It was found that elastins from mammal and bird (homeotherms) and from reptile and cartilagenous fish (poikilotherms) initiated coacervation at their respective ambient body temperatures, suggesting that this phenomenon may be involved in elastin fiber formation *in vivo*. Elastin from bony fish, however, which has the lowest content of hydrophobic and crosslinking amino acids, did not coacervate within a temperature range of 10–45°C. In addition, microcalorimetry revealed that elastins from aortae of higher vertebrates exhibited increased resistance to deformation and a greater magnitude of heat exchange upon extension and relaxation, as compared to those from lower vertebrates. A positive correlation between hydrophobic index and the change in internal energy for the different elastins suggests that a hydrophobic mechanism is involved in the elasticity of elastin at lower physiological extensions.

MATERIALS AND METHODS

Purification of Elastin

Elastin was isolated from the aortae of both vertebrates and invertebrates which represented major classes and/or phyla within the animal kingdom. In some cases other tissues were selected in addition to blood vessels. A complete listing of these animals, tissues, and methods of analysis can be found in Table 1 of Sage and Gray [2]. In the present report, these data have been summarized or, where appropriate, data from individual species have been selected.

Elastin was purified as previously described [2]. Very briefly, the procedure consisted of (1) defatting aorta minces in ethanol and ether, (2) extraction of milled tissue using 5.2 M guanidine hydrochloride containing 1% β-mercaptoethanol, and (3) autoclaving of the insoluble residue to remove collagen. Amino acid analysis was performed under standard conditions as described [2], and desmosine/isodesmosine content was evaluated by high voltage paper electrophoresis at pH 1.7, 3.5 (preparative), and 6.4, by ultraviolet spectral analysis, and by amino acid analysis [2].

The hydrophobic index of each elastin was calculated from the amino acid composition as reported by Sage and Gray [4], according to the formula:

$$\text{Hydrophobic Index, kcal/charged residue} = \frac{\text{average hydrophobicity}}{\text{fractional charge}}$$

The average hydrophobicity is a product of the free energy of unfolding for each hydrophobic amino acid and its corresponding mole fraction in the protein, and the fractional charge corresponds to the mole fraction of polar residues in the protein.

Histology

Tissues were fixed in 10% formalin and embedded in paraffin. Sections (4 μm in thickness) were stained using the Verhoeff Elastica stain (hematoxylin/ferric chloride/iodine) followed by a Van Gieson counterstain (acid fuchsin/picric acid) as previously described [3].

Coacervation

Elastin from pig, turkey, turtle, shark, black cod, and steel head aortae was solubilized by boiling in 0.25 M oxalic acid to produce α-(high molecular weight) and β-(low molecular weight) elastin peptides [8]. Oxalic acid was removed from the solubilized elastin by dialysis at 4°C for 24 hr against frequent changes of distilled water, followed by lyophilization. The lyophilized material was then dissolved in 2 ml of distilled water and passed through a Sephadex G-75 column (1.3 × 75 cm), equilibrated with 0.01 M acetic acid and calibrated with blue dextran (Pharmacia), albumin (Sigma), ovalbumin (Mann Chemical

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Co.), chymotrypsinogen A (Mann Chemical Co.) and NaCl. Absorbance was read at 280 mμ, and those peptides corresponding to a nominal molecular weight of approximately 50,000 and above (α-elastin) were pooled and lyophilized. The isoionic point of each α-elastin preparation was determined by measuring the pH of a solution containing the material after dialysis for one week against frequent changes of distilled water at 4°C.

Aliquots of α-elastin, dissolved in 0.01 M sodium acetate at pH 4.0, 5.0, and 6.0 (0.2–0.9 mg α-elastin/ml), were transferred to small test tubes and incubated one hour in a water bath at various fixed temperatures (10°C, 20°C, 29°C, 37.5°C, and 45°C). The tubes were then transferred to a fixed-angle rotor (IEC, Type No. A-321) which had been brought to the same temperature and centrifuged for 15 min at 20,000 *xg* in an IEC ultracentrifuge with a controlled temperature chamber. An aliquot of the supernatant for each pH and temperature was hydrolyzed and quantitated by amino acid analysis. Control figures were obtained from α-elastin samples incubated at 4°C. The amount of α-elastin remaining in solution at each pH and temperature was calculated for the various animal species studied.

Microcalorimetry

Aortae were extracted according to the procedure described above [2] but were treated as whole tissue sections which could be mounted and stretched in a microcalorimeter. The aortae, consisting of purified elastin, were stored in sterile water at 4°C.

The microcalorimeter used in this study was designed and built by Dr. J. Gosline (Department of Zoology, University of British Columbia, Vancouver, B.C.). The bath consisted of three concentric cylinders, each equipped with a stirrer and equilibrated with water at 17.6°C. The sample was air-dried, so that it lay straight, with the ends compressed to a few millimeters in width. Each end of the dried aorta was then glued with Epoxy cement into a brass mounting cup. The sample was rehydrated, its length was measured (10–15 mm), and the cups were mounted into a brass frame, the upper end of which was connected to a wire which was, in turn, attached to a bridge box containing a force transducer and strain gauge. When the gauge was turned to extend the sample a given length, the wire pulled on the mounted aorta and the force needed to stretch the sample was recorded. The aorta and its mount were immersed in a test tube filled with water, and the entire probe rested on a thermopile within the smallest cylindrical bath. The heat evolved when the aorta was stretched was recorded as a broad peak. The microcalorimeter measures the rate of heat production (dQ/dt). When the heat produced upon stretching is of sufficient magnitude over a relatively short period of time, the area under the peak can be taken to represent the total amount of heat produced, $Q_T = \int_{t=0} dQ/dt$. The system is reversible, in that relaxation of the aorta, causing heat to be absorbed, is recorded as a negative peak of the same size as that produced when the sample was first stretched. A complete description of this instrument and the calorimetric equations can be found in Gosline [6].

Each elastin sample was stretched at 3–5 different extensions, and the heat exchange and mechanical work for each extension were calculated from the imposed force and the heat evolved.

RESULTS

Distribution of Elastin

Table I summarizes the occurrence of elastin in the animal kingdom. Elastin was found to be strictly a vertebrate protein, although it was absent from cyclostomes (a group of primitive, jawless fish, to which the lampreys and hagfish belong). Since several other groups had reported the presence of elastin in both cyclostomes and invertebrates (see reference 2 for a review), we utilized multiple criteria to define elastin and to assess its presence in a tissue. These criteria were (1) amino acid analysis of a purified, insoluble residue after extraction of collagen and noncollagenous glycoproteins, (2) the presence of the crosslinking amino acids desmosine and isodesmosine, as identified by high voltage electrophoresis, ultraviolet absorption spectra, and amino acid analysis, and (3) a positive reaction with the Verhoeff Elastica stain. There was complete consistency among all the criteria used for each elastin sample; in no instance was a protein observed with an elastin-like amino acid composition that failed to contain crosslinks or that did not react with the Verhoeff stain. Elastins from different vertebrates displayed marked interspecies variations, however, which were reflected in (1) the amounts of elastin relative to total protein in comparable sections of aortae (Table I), (2) the amino acid compositions, especially with respect to hydrophobic and crosslinking amino acids, and (3) the morphology and distribution of elastin-containing fibers. These differences, and their relationship to the phemonena of coacervation and elasticity, are discussed below.

TABLE I. *Phylogenetic distribution of elastin*[a]

Animal group	Histology[b]	% of Tissue protein[c]
I. Vertebrates		
Mammal	+++	56
Bird	+++	50
Reptile	++	33[d]
Amphibian	+	8
Fish		
a. Teleost	+	30
b. Nonteleost	++	10
Jawless Fish	—	<0.03[e]
II. Invertebrates	—	<0.50[e]

[a] In the aortae; data from Sage and Gray [2].
[b] Based on the Verhoeff elastica stain.
[c] Based on the ratio of [Des + Ide] in the unfractionated tissue to [Des + Ide] in the purified elastin.
[d] Based on the amount
after extraction.
[e] Upper limit of elastin content in these vessels; the amino acid composition of the purified insoluble material did not resemble that of elastin.

Comparative Histology

The abundance and distribution of elastin in vessels from a mammal (pig), bony fish (catfish, a teleost), non-teleost (gar, an holostean), and a jawless fish (lamprey, a cyclostome) can be visualized at low magnification in Fig 1. Each of these vessels receives the immediate ventricular output and represents an adaptation in each animal for damping of pressure pulses, thereby ensuring an even flow of blood to the systemic circulation (mammal) or to the gill capillaries (fish). The pig aorta contained numerous, densely packed elastin fibers arranged in concentric lamellae (Fig 1*A*). This arrangement was similar in gar, although the lamellae appeared finer and were restricted to a smaller area of the vessel (Fig 1*C*). Elastic fibers in the bulbus arteriosus of the catfish were highly convoluted and asymmetric (Fig 1*B*), while they appeared to be totally absent in the jawless fish aorta (Fig 1*D*).

Further details of elastic lamellae can be seen in Fig 2 and 3. These fibers were similar in aortae from chicken and 2 nonteleost fish (sturgeon, a chondrostean; shark, a primitive cartilagenous fish) and appeared to be finer and more wavy than those from pig aorta, which exhibited fewer infoldings and presented a highly regular pattern (Fig 2, compare *panel A* with *B–D*). Additional variations can be seen among ventral aortae from an amphibian and other nonteleost fish (Fig 3); in these animals, it is the ventral aorta which is interposed between the heart and the gills. The nature of the inclusions in the salamander aorta and in that of the air-breathing lungfish, which is thought to represent an early tetrapod ancestor, is not known (Fig 3, *A* and *C*). The elastic fibers in the aorta of the coelacanth, a form once thought to be extinct and from which the tetrapod line also evolved, were exceedingly fine but gave the impression of being arranged in lamellar units (Fig 3*B*). The most delicate, reticular elastic network was observed in the aorta of a paddlefish, a primitive chondrostean fish (Fig 3*D*).

Several interesting variations in the disposition of elastin-containing fibers were seen among teleost fish (Fig 4). The ventral aorta of the walleye (Fig 4*C*) revealed the convoluted elastic fiber arrangement which was seen at low magnification in the catfish (Fig 1*B*). Examination of the mesenteric vessels of a filefish showed an exclusive distribution of elastin in an area corresponding to the internal elastic lamina of higher

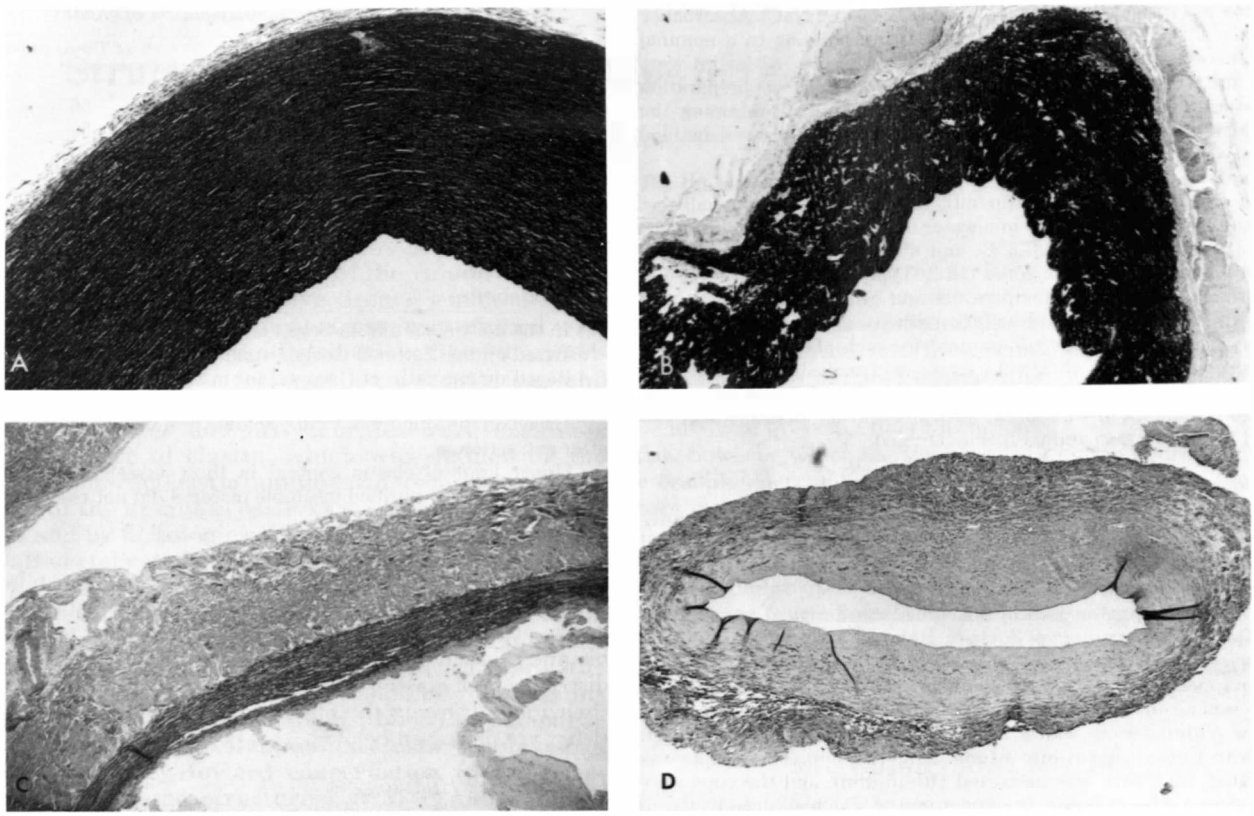

FIG 1. Distribution of elastic fibers in vessels receiving direct output of ventricle (× 32). *A*, Pig, thoracic aorta; *B*, catfish, bulbus arteriosus; *C*, gar, conus arteriosus; and *D*, lamprey, conus arteriosus. (From Sage and Gray [3], with permission.)

vertebrates; however, sectioning of this material at an oblique angle revealed a highly compressed, folded membrane (Fig 4, *A* and *B*). The cyclostome aorta, viewed at higher magnification, appeared devoid of elastin-containing fibers (Fig 4*D*, compare with Fig 1*D*).

Amino Acid Composition and Hydrophobic Index

In Table II are listed the amino acid compositions of purified elastins from different vertebrate groups. A representative has been selected from several major classes which most closely conforms to the general compositional characteristics of the group as a whole. A complete tabulation can be found in Sage and Gray [2].

As the most hydrophobic protein known, elastin exhibits several unique compositional features (see reference 9 for a review). If pig aortic or bovine ligamentum nuchae elastin is used as a reference standard, the protein contains approximately 5% polar amino acids, 2–3 desmosine plus isodesmosine residues/1,000 residues, low levels of hydroxyproline, and a significant enrichment in four amino acids: glycine (33%), alanine (24%), valine (12%), and proline (12%).

It can be seen in Table II that the overall amino acid compositions of avian and reptilian elastin resembled that of mammalian elastin, although decreases in alanine and phenylalanine accompanied by increased levels of valine and proline were evident. There was considerably more variation among the fish proteins. In general, elastin from nonteleosts (shark, chondrosteans, holosteans, lungfish) exhibited lower contents of alanine, valine, and phenylalanine, but some were distinguished by very high levels of tyrosine and glutamic acid/glutamine. The teleost group formed the most distinct subset of elastins, however, in that markedly reduced amounts of hydrophobic amino acids, including proline, alanine, valine, leucine, and isoleucine, were accompanied by an increase in certain specific polar amino acids such as arginine and threonine. In addition, the average glycine content was 41%, desmosine + isodesmosine averaged 0.9 residues/1,000 residues [4].

The hydrophobic index was calculated for the various elastins and has been presented as an average value for several vertebrate groups in Table III. For a more complete listing of individual species, the reader is referred to Sage and Gray [4]. Avian elastin appeared the most hydrophobic, and elastin from fish was found to be the least hydrophobic. The contribution of fractional charge (see Materials and Methods) resulted in a low value for the hydrophobic index of non-teleost elastin. It was of interest that a general increase in blood pressure, which occurred during vertebrate evolution, could be correlated with an increase in the hydrophobic index of elastin (Table III).

Coacervation Studies

Limited hydrolysis of insoluble elastins from several vertebrates with 0.25 M oxalic acid solubilized the proteins at different rates. The teleost (steelhead and black cod) elastin was dissolved entirely after 3 extractions while that of the shark and turtle required 5. Pig and turkey elastin did not become totally solubilized even after 7 extractions.

The elution profiles from Sephadex G-75 of the oxalic acid-treated elastins revealed a large peak containing α-elastin (M_r > 50,000) in the digests of higher vertebrate elastin (data not shown). The α and β (M_r < 50,000) fractions were more equally divided in the shark elastin. Black cod elastin showed an equal distribution of α and β material, while elastin from the steelhead contained a higher proportion of the β component. In all cases, the separation between α and β components was distinct. The α-elastin from each of these species was used for the coacervation experiments.

To check whether any specific regions were selectively solubilized by the oxalic acid digestion, amino acid compositions of the α and β fractions were determined for all of the species.

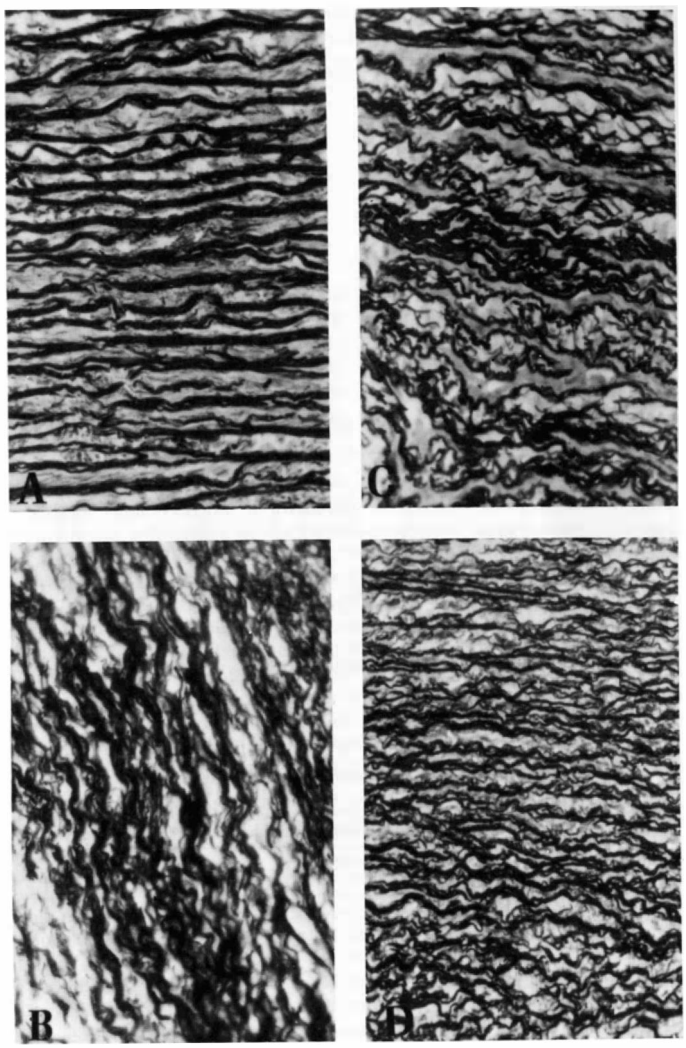

FIG 2. Morphology of elastic lamellae in aortae of four different vertebrates (reduced from × 324). *A*, Pig, dorsal aorta; *B*, sturgeon, ventral aorta; *C*, chicken, dorsal aorta; and *D*, shark, ventral aorta. (From Sage and Gray [3], with permission.)

There were no major differences among α and β-elastin and purified insoluble elastin. It was therefore concluded that the α-elastin fraction contained all the domains represented in intact elastin chains.

The isoionic points of the α-elastins were close to pH 5 in all cases; the pH range chosen for coacervation accordingly was from 4 to 6. In using pH 5 as the isoionic (isoelectric) point, we have assumed that no ions other than H^+ and OH^- were bound to the α-elastin.

The absolute solubilities of α-elastin at pH 5 in 0.01 M sodium acetate at 25°C varied from 26 mg/ml for the pig to 12 mg/ml for the steelhead. These figures were calculated by determining the concentration from amino acid analysis of a saturated solution of the α-elastin which had equilibrated three days at 25°C. The concentrations used for the coacervation experiments represented a similar degree of saturation for all the samples and were comparable to the absolute solubility concentrations in all cases except that of the steelhead, which was slightly lower. Since the usual concentration of pig and bovine α-elastin used for coacervation is 0.1 mg/ml [7], the solutions in the present study were well within the range for optimal coacervation.

A cloudy precipitate was observed at 29°C, 37.5°C, and 45°C in tubes containing α-elastins from all species except the teleost fish. The proportion of α-elastin which remained in solution (uncoacervated) was measured by amino acid analysis and was expressed as percent of soluble protein at the control temperature of 4°C (Fig 5). At 37°C, the teleost α-elastin did not coacervate but the α-elastin from the other species showed from 40% and 80% coacervation. More protein coacervated at pH 6 than at pH 4, but pH 5 was the optimum pH for this process.

Turtle α-elastin appeared to coacervate at a lower temperature than did pig or turkey α-elastin; shark α-elastin began coacervating at an even lower temperature than that seen for the turtle protein. Its apparent dissolution at higher temperatures could be indicative of a transition point at which increasing heat drives coacervated material back into solution. The small amount of protein which coacervated in the steelhead α-elastin was checked by amino acid analysis. There was no detectable difference in composition between the α-elastin, uncoacervated material, and material which had apparently coacervated at 37.5°C and 45°C.

Microcalorimetry

Intact aortae from 6 vertebrates were subjected to the purification procedure for elastin. Amino acid analyses of the residues compared closely with the analyses of elastins purified from minced aortae, suggesting that collagen and soluble proteins had been almost completely extracted. Carp "elastin,"

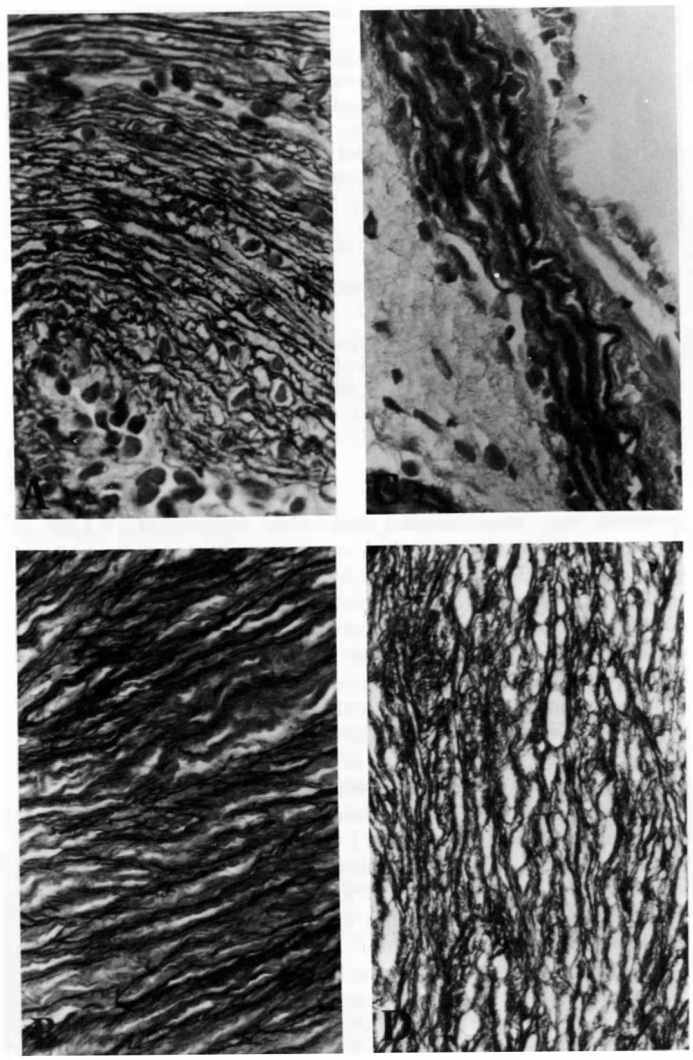

FIG 3. Morphology of elastic lamellae in ventral aortae of amphibian and fish (reduced from × 324). *A*, Salamander; *B*, coelacanth; *C*, lungfish; and *D*, paddlefish. (From Sage and Gray [3], with permission.)

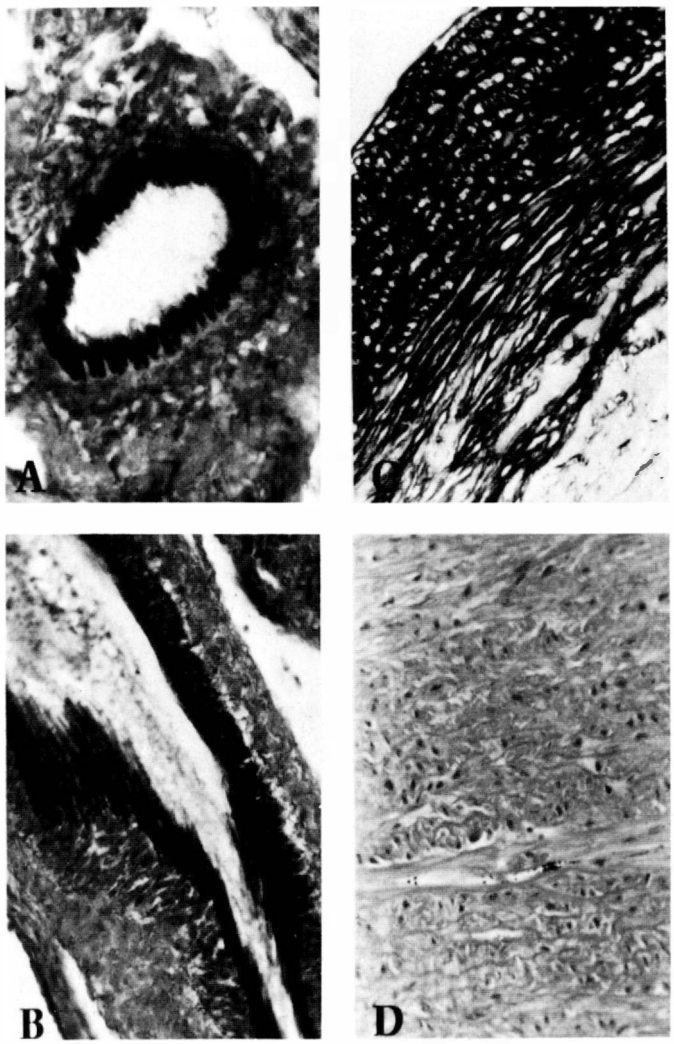

FIG 4. Distribution of elastin in fish vessels. *A*, Filefish, mesenteric artery (reduced from × 324). *B*, filefish, mesenteric artery (reduced from × 809). *C*, walleye, ventral aorta (reduced from × 324). *D*, hagfish, ventral aorta (reduced from × 324). (From Sage and Gray [3], with permission.)

TABLE II. *Amino acid compositions of aortic elastins from selected vertebrate groups*[a]

	Mammal[b]	Bird[c]	Reptile[d]	Teleost[e]	Nonteleost[f]
Lys	5.2	4.8	6.8	5.4	11
His	1.0	1.6	3.6	2.2	2.4
Arg	7.9	6.9	7.6	17	15
Asx	6.4	3.7	3.4	12	17
Thr	15	11	18	60	31
Ser	12	5.5	11	29	12
Glx	19	17	24	27	61
Hyp	8.7	11	16	7.3	3.6
Pro	113	136	130	98	128
Gly	313	338	319	449	312
Ala	244	190	184	136	152
Val	128	154	151	47	97
Ile	18	21	17	7.7	19
Leu	54	53	58	29	59
Tyr	19	15	34	36	64
Phe	33	24	13	33	9.9
Cys	<1	2.3	<0.9	1.2	<0.6
Met	<1	0.9	2.0	3.5	4.6
Ide	1.9	1.4	1.5	0.3	0.9
Des	1.3	1.0	1.2	0.3	0.9

[a] In residues/1,000 residues; data from Sage and Gray [2].
[b] Pig; aortic arch.
[c] Goose; aortic arch.
[d] Turtle; aortic arch and descending aorta.
[e] Black cod; bulbus arteriosus
[f] White sturgeon; ventral aorta.

TABLE III. *Hydrophobicity of elastins*[a]

Animal group	Hydrophobic index, kcal/charged residue[b]	Representative Blood Pressure[c]
Mammal	23	120/80
Bird	33	149/43
Reptile	24	80/60
Amphibian	16	30/20
Fish		
a. Teleost	11	29/18
b. Nonteleost	9	26/19
Jawless fish	—	5/3

[a] Data from Sage and Gray [4].
[b] Average value calculated from hydrophobic indices of all representatives within a vertebrate class, as shown in (reference 4, Table II).
[c] Systolic/diastolic pressure, mm Hg [10].

however, was contaminated with other components, based on the low glycine and crosslink content and elevated levels of polar amino acids in the insoluble residue. Since it was important to have as pure an elastin preparation as possible, the contribution of other components to the elastic response, especially in the case of carp aorta, must be considered. At the low extension ratios used, however, it was unlikely that any other protein was interfering with elastin. The quantities measured in these experiments are actually a reflection of the resistance to deformation typical of rubbery materials, and it is elastin which provides this resistance at low extensions.

Table IV lists values for the heat exchange (Q) and mechanical work (W) for the different aortae after an extension and subsequent relaxation of samples stretched 30% of their original length. The process was reversible except in the cases of sturgeon conus arteriosus, tarpon bulbus arteriosus, and possibly turtle aorta, which showed more complete reversibility at lower extension ratios. In all cases the heat produced upon stretching the sample exceeded that corresponding to the mechanical work involved. The ratio of these heats, W/Q, was much higher for the fish than that corresponding to the higher vertebrates.

Force-extension and heat-extension curves for some of the elastin samples are shown in Fig 6. The extension ratio was calculated as stretched length/original length, i.e., 1.3 is the equivalent of a 30% extension. The slopes of the force-extension and heat-extension curves corresponding to one sample were very similar in all cases but the sturgeon conus arteriosus. In this instance, a significantly greater amount of force was required to elicit a similar magnitude of heat exchange when compared to tarpon BA (Fig 6).

When the W/Q ratio was plotted as a function of extension ratio, the relationship between these two parameters was found to be linear for all the elastins (data not shown). Elastins from the three higher vertebrates were clustered in the same general area, while those from carp and sturgeon were located in another distinct region which reflected the higher W/Q values for these species. The plot suggested that the mechanics involved in stretching aortae, which were composed mostly of purified elastin, were different for higher and lower vertebrates. The variations in the magnitudes of heat exchange and force were difficult to standardize, due to differences among vessel architecture and sample size, but the quantity W/Q for a particular extension should be characteristic for each aorta and should provide a reasonable basis for comparison among different aortae.

The relationship between W/Q and hydrophobic index for the various elastin samples was examined in Fig 7. There was a strong negative correlation between W/Q and the hydrophobic index. This observation suggested that the production of heat, as resistance to stretching, was related to the hydrophobicity of the elastin. In addition, the absolute difference between

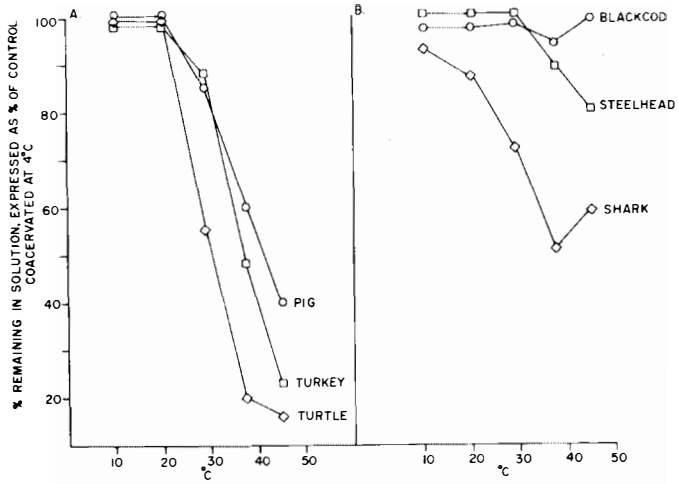

FIG 5. Coacervation of α-elastins at pH 5.0. Elastin was purified from aortae, solubilized by oxalic acid treatment, and chromatographed on Sephadex G-75, as described in "Materials and Methods." Fractions corresponding to α-elastin (M, > 50,000) were pooled and subsequently used for coacervation studies. Aliquots (0.2–0.9 mg α-elastin/ml) were incubated 1 hr at the temperatures indicated. Coacervated material was collected by centrifugation at the given incubation temperature. Vertical axes represent the extent of coacervation of the sample, as measured by amino acid analysis of the supernate, and expressed as percent of control α-elastin which coacervated at 4°C. *A*, Homeothermic vertebrates; *B*, poikilothermic vertebrates.

TABLE IV. *Heat exchange (Q) and mechanical work (W) when elastin samples are extended and relaxed*[a]

Sample	W, mcal	Q (extension)[b], mcal	Q (relaxation)[b], mcal	W/Q
Pig[c]	0.30	−2.8	+2.4	0.11
Goose[c]	0.28	−3.0	+2.8	0.09
Turtle[c]	0.14	−1.8	+1.4	0.08
Tarpon BA[d]	0.11	−0.61	+0.40	0.18
Carp BA[d]	0.03	−0.14	+0.12	0.24
Sturgeon VA[e]	0.09	−0.33	+0.33	0.27
Sturgeon CA[f]	0.21	−0.57	+0.35	0.29

[a] Samples were stretched 30%, in water, at 17.6°C.
[b] + denotes heat absorbed by the system; − denotes heat released by the system.
[c] Elastin samples were purified from dorsal aorta.
[d] Bulbus arteriosus.
[e] Ventral aorta.
[f] Conus arteriosus.

the heat exchange and mechanical work (Q-W) was significantly greater for the more hydrophobic elastins (Table IV and Fig 7).

Relationship of Elastin to Other Connective Tissue Proteins

The amino acid compositions of present-day elastins that were isolated from representatives of each vertebrate class did not resemble those of other connective tissue proteins, including collagen types I–V, silk fibroin, keratin, or elastin microfibrillar glycoprotein [4]. In addition, it did not indicate homology with respect to 3 other elastic proteins: resilin, found in insects [11], abductin, a molluscan hinge protein [12] and elastic fibers from *Octopus* [13]. However, it was possible that elastin and other structural, connective tissue proteins, especially those with repetitive amino acid sequences, arose by divergence from a common ancestral gene. Accordingly, an amino acid composition for an hypothetical, ancestral elastin was reconstructed from the existing compositional data for the present-day elastins [4]. Selected features of this composition have been listed in Table V, along with the amino acid compositions of mammalian elastin, type I collagen, and the microfibrillar component. It can be seen that the ancestral elastin did not differ greatly in composition from the mammalian elastin, although

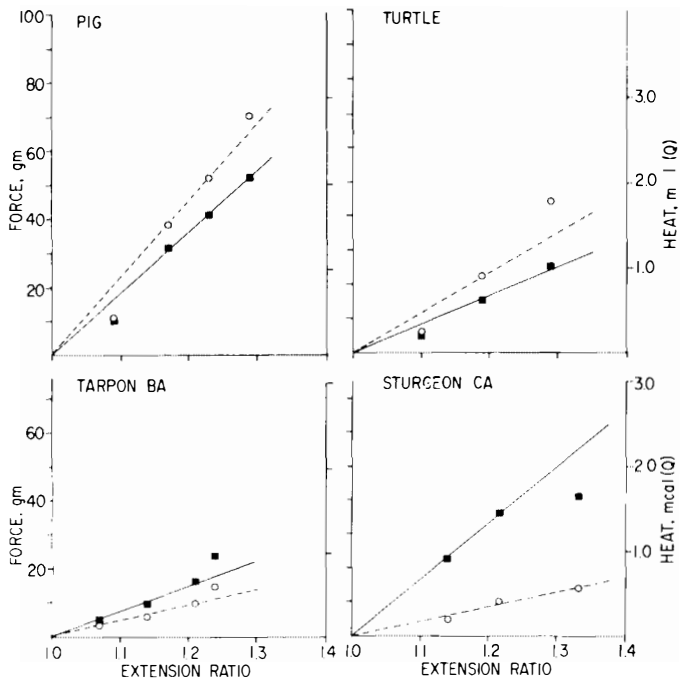

FIG 6. Force-extension and heat-extension curves for samples of elastin stretched in water at 17.6°C. Insoluble elastin was prepared from dorsal aortae (*pig*, *turtle*), bulbus arteriosus (*tarpon*), and conus arteriosus (*sturgeon*), and these samples were stretched in a microcalorimeter, as described in "Materials and Methods." Stress, as measured by force transduction (-■-), and heat exchange (-○-) were plotted as a function of extension ratio (the ratio of extended length to starting length) for four different vertebrates, as shown.

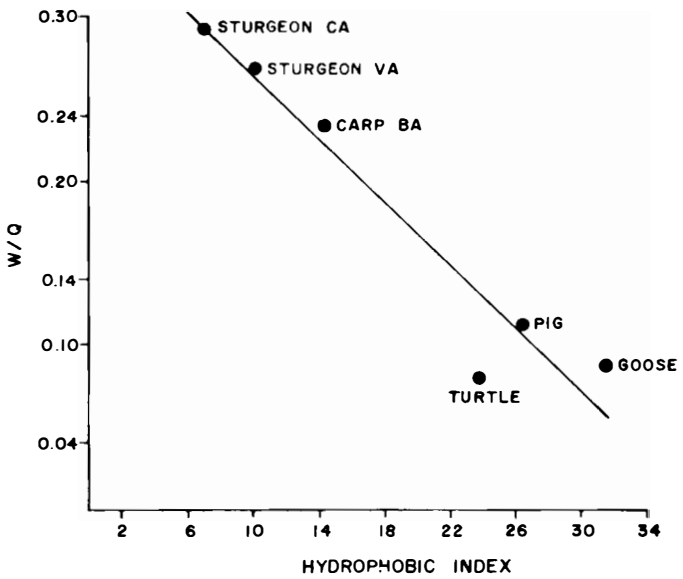

FIG 7. Relationship between W/Q and hydrophobic index for elastin samples stretched in water at 17.6°C. Values of W/Q for samples stretched 30% (Table IV) were plotted against the hydrophobic index corresponding to that particular elastin (Table III, and Sage and Gray [4]). A negative correlation can be seen between the degree of hydrophobicity of the elastin and the magnitude of the W/Q ratio.

it was significantly less hydrophobic and contained reduced amounts of alanine accompanied by higher amounts of polar amino acids. In spite of an apparent similarity between the ancestral elastin and collagen with respect to certain amino acids (glycine, alanine, glutamic acid plus glutamine), structural studies on both mammalian and avian tropoelastin have not revealed homologous sequences between these proteins and

TABLE V. *Comparison of the amino acid compositions of elastin and other connective tissue proteins*[a]

	Ancestral elastin[b]	Elastin[c]	Type I Collagen[d]	Microfibrillar glycoprotein[e]
Hyl		NP	5.8	NP
Lys		3.8	27	31
His		NP	4.8	14
Arg		3.6	51	42
Asx		2.8	45	120
Thr	30	4.8	18	56
Ser	25	5.6	36	65
Glx	70	15	73	117
Hyp		6.4	94	NP
Pro		149	128	69
Gly	310	331	330	123
Ala	130	249	110	49
Val	100	106	25	46
Ile		20	9.5	47
Leu		54	24	50
Tyr	40	17	2.8	31
Phe	20	20	12	30
Cys		NP	NP	88
Met		NP	6.2	15
Ide + Des	2.5	2.8	NP	NP
Hydrophobic Index	10	20		

[a] In residues/1,000 residues.
[b] Based on reconstruction of the nodal amino acid composition for selected residues from an hypothetical vertebrate ancestor [4].
[c] Human skin [14].
[d] Human skin [15].
[e] Bovine ligamentum nuchae [16].
NP = not present.

collagen (reference 17 and references therein). The ancestral elastin also did not resemble the microfibrillar protein(s) or other elastic proteins (Table V; [4]).

DISCUSSION

Characterization of elastins purified from aortae of numerous vertebrate species has resulted in several observations regarding the evolution of this rubbery protein: (1) Elastin first appeared in an early Devonian ancestor of present-day fish, at some point after the divergence of the cyclostome (jawless fish) and gnathostome (jawed fish) lines. (2) Comparison of the amino acid composition of an hypothetical ancestral elastin with compositional data from other structural, connective tissue proteins did not reveal a common genetic origin for these proteins. (3) There was marked interspecies heterogeneity with respect to amino acid composition among the elastins; these compositions were highly suggestive of an evolutionary trend in the vertebrates which favored selection of an increasingly more hydrophobic elastin. In addition, such differences could be indicative of distinct genetic types of elastin. (4) There was also heterogeneity in elastin fiber morphology and disposition among vertebrate aortae; in general, animals with higher blood pressures possessed a greater number of well-formed, concentric elastic lamellae in their arteries. (5) Preparations of α-elastin coacervated in the range of ambient body temperature for homeotherms and for certain poikilotherms; however, the more polar teleost elastins did not exhibit this property. (6) Lastly, there appeared to be a relationship between the elastic behavior of elastin and its hydrophobicity, suggesting that the hydrophobic character of elastin at least partially determines the response of arteries to stress.

The existence of distinct genetic types of elastin was suggested by several studies which demonstrated compositional and limited sequence variability among insoluble elastins purified from different tissues [18–20]. In addition, recent studies on elastin biosynthesis using cell-free translation of embryonic chick lung and aortic mRNA have revealed two polypeptides which could represent distinct genetic types [23]. There has been disagreement regarding the uniqueness of elastins in different tissues from the same animal species, however [21,22]. Studies from this laboratory [2] and from others [24,25] have described an elastin in teleost fish aorta which was compositionally distinct from elastins in aortae of other vertebrates. Identification of this protein as an elastin type which is different from the predominant type found in mammals, however, must await further biochemical characterization.

The temperature-dependent, reversible phase separation which occurs with solutions of soluble α-elastin, and with tropoelastin, was first described in detail by Partridge, Davis, and Adair [8]. The present study, which utilized α-elastins of different hydrophobic indices from both homeotherms and poikilotherms, investigated (a) the involvement of hydrophobicity in coacervate formation and (b) the significance of coacervation as a step in elastin fibrillogenesis *in vivo*.

It has been suggested that hydrophobic interactions are the driving force which causes synthetic polypeptides of elastin and tropoelastin molecules to coacervate [7,26]. The polymerization behavior (coacervation) of Bence-Jones proteins at higher temperatures has also been attributed to hydrophobic interactions [27]. The present results indicate that α-elastins with very high hydrophobic indices (pig, turkey, turtle) coacervated at elevated temperatures. Black cod α-elastin and steelhead α-elastin, which have markedly lower hydrophobic indices [4], did not coacervate significantly. In contrast, shark α-elastin, which also has a low hydrophobic index (approximately 10 Kcal/residue), coacervated at the lowest temperature of all the elastins (Fig 5). It may be that all α-elastins are sufficiently hydrophobic to undergo polymerization at elevated temperatures, and that another factor is involved in the process. Alternatively, the hydrophobic index may not be accurately representing the actual nonpolar contribution of the side chains of some elastins. Shark and mammalian elastin have very similar levels of hydrophobic amino acids; consequently, their average hydrophobicities (a measure of only the apolar residue mole fraction) are almost identical. However, shark elastin has twice to 3 times the fractional charge value found in mammalian, bird, and reptilian elastin due to its high content of glutamic acid [2,4]. Therefore, its hydrophobic index $\left(\frac{\text{average hydrophobicity}}{\text{fractional charge}}\right)$ appears much lower. The average hydrophobicity of teleost elastin, however, is significantly lower than that of elastin belonging to any other group. The data suggest that elastins of similar composition coacervate, while those from teleost fish, which have reduced valine and alanine contents, do not display this tendency to a significant degree. Since tropoelastin also coacervates [7], the low crosslink content of the teleost elastins was probably not a contributing factor to the apparent absence of this phenomenon among them.

Results from electron microscopic and optical diffraction studies have suggested that elastin fibers are formed *in vivo* by the process of coacervation, as the periodicity observed in filaments of tropoelastin coacervates was similar to that of mature crosslinked elastin [7]. If this theory is correct, the elastin of cold-blooded animals should coacervate at lower temperatures than that of warm-blooded animals. Elastins from 2 of the poikilotherms (black cod and steelhead) did not appear to utilize this mechanism at their ambient temperatures. The shark and turtle α-elastin, however, did exhibit decreased solubility at lower temperatures. The average body temperature of both hammerhead shark (18°C) and green sea turtle (20–30°C) correlated closely with the coacervation temperature of the respective α-elastins (Fig 5).

Recent studies by Gosline and co-workers [6,28,29] have clarified several points on the elastic mechanism of elastin. The elastic properties of this protein are similar to those of rubber, which combines a high extensibility with a low modulus of elasticity. The resilience of the aorta is due to the property of

time-independent, linear elasticity which allows elastin to store energy reversibly with low hysteresis. The kinetic theory of rubber elasticity depicts elastin as randomly coiled, kinetically free, crosslinked polymer chains which are in a state of maximum entropy. When the polymeric network is stretched, the system becomes more ordered, resulting in a decrease in conformational entropy. The tendency for systems to be in a state of maximum entropy provides energy for recoil of the elastin chains.

In addition, hydrophobic interactions have been implicated in the elastic mechanism of elastin [5]. Gosline [6] has recently proposed that the temperature-dependent internal energy change which accompanied the stretching of elastin fibers could be explained by the adsorption of water onto hydrophobic groups of the protein. The β-spiral domains of elastin, which contain mostly hydrophobic residues, are thought to function in such a solvent-polymer interaction [30].

From Fig 6 it can be seen that the force-extension and heat-extension curves were nearly linear over the range in which the aortae were stretched, in agreement with results obtained using ligament elastin [5] and salmon arterial elastin [25]. In all samples, the heat (Q) produced during stretching in water exceeded that produced by the mechanical work (W) involved (Table IV), although proportionally less heat was evolved for a given amount of mechanical work in the case of fish elastins. The ratio W/Q was therefore highest for fish elastin. The process was nearly reversible at lower extension ratios, but at 30% extension sturgeon conus arteriosus and tarpon bulbus arteriosus did not recover all the heat upon relaxation which had been evolved during stretching. These structures may have a slow component which functions at higher extensions, or the elastin may have started to creep in this range. Fig 7 illustrates the relationship between W/Q and the hydrophobic index for each elastin studied. There was a strong negative correlation between the hydrophobic index and the ratio of mechanical work to heat evolved at low extension ratios. The difference between the heat exchange and mechanical work (Q–W) is equal to the change in internal energy for the sample. This difference was much greater for more hydrophobic elastins with samples of approximately the same dimensions and elastin content. An increase in the hydrophobicity of elastin apparently occurred with the development in higher vertebrates of elevated blood pressures and more efficient circulatory systems. The arterial walls of higher vertebrates were found to be much stiffer than those of the fish, with an increased concentration of elastic lamellar units. A less compliant aorta would offer more resistance to volume changes during pressure fluctuations, thereby maintaining a higher tension on the arterial wall and a more highly pressurized blood flow in the smaller vessels.

Gosline and French [28] have, in addition, suggested that the hydrophobic properties of elastin provided selective pressure toward the evolution of a temperature-independent elastic protein in lower, poikilothermic vertebrates. Examination of two properties of elastin for which hydrophobic interactions have been claimed to be responsible lends additional support to this selection hypothesis. If coacervation is a mechanism by which elastin fibers are formed *in vivo*, and the driving force of this process is hydrophobic interactions, then an increasing hydrophobicity should favor the dense fiber arrangements, e.g., the elastic lamellae, which are seen in the higher vertebrates. This study suggests that elastin fibers, with the exception of those in teleost fish, may form by a type of coacervation process. In addition, the magnitude of the W/Q ratio was shown to decrease concomitantly in elastins of increasing hydrophobicity.

Elastin is the most hydrophobic protein known. This characteristic has undoubtedly evolved as a result of strong selective pressures favoring the optimal functioning of this protein under different physiological conditions.

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